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BCSH REVIEW

Use and evaluation of leucocyte monoclonal antibodies in the diagnostic laboratory: a review

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Summary The diagnosis of haematopoietic disorders by immunological methods, termed 'immunophenotyping', or 'cell marker analysis', is now a routine investigation in many haematology laboratories. A wide range of antibodies and fluorochromes are commercially available, in addition to antigen detection systems: immunocytochemistry, direct or indirect immunofluorescence, incorporating either light microscopy or flow cytometry. In view of this variety in methodology, it is important that all antibodies are used appropriately. The following review highlights a variety of issues, including antibody selection and dilution, negative controls, storage, fixatives as well as quality control, that require consideration prior to the routine use of antibody reagents.

Keywords Immunophenotyping, monoclonal antibodies, quality control, flow cytometry

Introduction

Hybridoma technology, developed in the mid-1970s by Köhler and Milstein (1975), made it possible to produce virtually limitless quantities of highly specific monoclonal antibodies (mAbs) that recognize distinct epitopes of cellular antigens. There is currently available a large number of such reagents (greater than 1100) which detect different molecules on both normal and neoplastic cells from the various lineages. In 1982 at the 'First International Workshop on Leucocyte Differentiation Antigens', the term 'Cluster of Differentiation' (CD) was introduced to help classify antibodies detecting antigens with similar tissue distribution. Five workshops have now been held and 130 CD groups established, a number that is likely to increase in the future. Only a minority of these reagents, however, are of value for the routine diagnosis of leukaemic disorders. It should be stressed that, to date, no leukaemia specific antibodies have been produced, and that all antibodies in diagnostic use recognize antigens which are also present on normal cells. It is for this reason that panels of antibodies, including both monoclonal and polyclonal reagents, are needed to obtain diagnostic information. In addition, determination of peripheral blood lymphocyte subsets, by immunological techniques, has been shown to be useful in the evaluation of immune deficiencies and autoimmune disorders and for monitoring transplant rejection therapy.

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Antibodies may be of different immunoglobulin class (e.g. IgG/IgM), subclass (e.g. IgG_1/IgG_2) and isotype (e.g. IgG_{2a}, IgG_{2b}). They can also be unconjugated, or conjugated to different fluorochromes (e.g. fluorescein isothiocyanate [FITC], tetramethylrhodamine isothiocyanate [TRITC], phycoerthyrin [PE], peridinin chlorophyll protein [Per-CP], PE-Cy5 and PE Texas Red) enabling multi-colour analysis. Newer and more sensitive fluorochromes are currently being developed. In addition to the wide variety of antibodies and fluorochromes, there are a number of different techniques available for the detection of cellular antigens including direct and indirect immunofluorescence, using either flow cytometry or microscopy, on whole blood or separated cells. In most laboratories immunocytochemical techniques are used for cytoplasmic antigen detection, although the recent availability of permeabilizing agents (e.g. PermeaFix-Ortho) will increase the use of flow cytometry in this area. In view of this variety of methodology it is vital that each antibody is correctly evaluated, for each individual technique, prior to its routine clinical use. The British Committee for Standards in Haematology has drafted guidelines for the minimum antibody panels required for the immunophenotyping of both acute and chronic leukaemias (British Committee for Standards in Haematology 1994a, 1994b). These documents do not give details on the selection and evaluation of antibodies and give only brief technical details on the use of antibodies.

The following review, incorporating results from the UK NEQAS (United Kingdom National External Quality Assessment Scheme) in Leucocyte Immunofluorescence and Immunocytochemistry, highlights the practical procedures

that should be considered in a laboratory's evaluation and use of antibody reagents.

Designation

The introduction of the cluster of differentiation (CD) system for categorizing mAb reactivity provides a consistent method for evaluating and comparing antibodies. All antibodies designated as reacting against a CD antigen should have been evaluated in one of the five international workshops on 'Human Leucocyte Differentiation Antigens' (Bernard *et al.* 1984; Reinherz 1986; McMichael 1987; Knapp 1989; Schlossman *et al.* 1994). However, discrepant data in the literature may, in part, be attributed to the use of different commercial sources, and hence clones, of a particular mAb (Reilly, Granger & Barnett, 1995). Unevaluated research and/or locally produced antibodies, i.e. those which have not been officially recognized as reacting against a cluster of differentiation, should generally be avoided in clinical diagnostic work. This does not apply to antibodies, such as FMC7, anti-myeloperoxidase and anti-TdT, that bind to antigens that have yet to be assigned a CD number.

Antibody labelling

Cells directly labelled with FITC conjugated antibodies will not be as bright as those stained using indirect FITC methods. Shapiro (1988) reported that the decreased sensitivity may be as much as five- to sixfold. This is of practical importance when the antigen expression is low. PE and the newer fluorochromes (e.g. tandem colour fluorochromes) have a much higher quantum yield than FITC, thus increasing sensitivity. The UK NEQAS in leucocyte immunofluorescence has consistently shown statistically significant differences between samples analysed with FITC and PE conjugated antibodies for the following antigens: CD3, CD5, CD13, CD14, CD33. For example, in a recent survey investigating CD13 detection, eight of 24 laboratories who used FITC conjugated antibodies obtained values of less than 50% (overall mean 58%), of which three were negative results, defined as less than 20% (British Committee for Standards in Haematology, 1994a). In contrast, all 12 laboratories using PE conjugated reagents obtained results greater than 50% (mean 77%). Therefore, when available, PE conjugates, or tandem colour fluorochromes, should be used for single colour analysis. In the case of two-colour analysis (see below) the more sensitive fluorochrome conjugated antibody should be used for the mAb detecting the weaker antigen.

Multi-colour analysis

Two-colour immunofluorescence has become widely accepted as the method of choice for analysing normal and leukaemic cells (Shapiro 1988; Uckun *et al.* 1989). It is an ideal method for demonstrating the co-expression of antigens in cases of biphenotypic leukaemias, particularly when the sample contains a mixture of normal and leukaemic cells. In addition, two-colour analysis is the recommended technique for immune monitoring, i.e. for quantitation of CD3+CD4+ and CD3+CD8+ cells (Centres for Disease Control, 1992). Single colour analysis will consistently over estimate both populations by up to 5%, and significantly more in cases with a high CD3−CD8+, NK population. Care is also required in the selection of antibody combinations to ensure the availability of appropriate (i.e. class, subclass, isotype) negative control reagents. It is possible that in certain two-colour combinations one antibody may prevent the binding of the second. This occurs when both antibodies (one of which has a higher avidity) compete for spatially adjacent sites (e.g. CD3 and the T cell antigen receptor complex). Finally, the development of fluorochromes, in addition to PE and FITC, that can be detected with a single laser, and the availability of computer software capable of rapid data analysis, have enabled the incorporation of triple-colour analysis in routine leukaemia diagnosis and detection of minimal residual disease (Campana, Coustan-Smith & Janossy 1990). However, this may lead to further technical problems, including more complicated spectral compensation, as well as the need for added expertise for instrument set up, data collection and analysis.

Reagent selection

There are a number of factors which require consideration in the choice of reagents. The nuclear enzyme terminal deoxynucleotidyl transferase (TdT) is best detected by immunofluorescence, or immunocytochemical techniques, using immunoadsorbed-purified polyclonal antibodies. Monoclonal anti-TdT antibodies are not always as sensitive as polyclonal reagents (Lanham *et al.* 1986) and are best used as cocktails. In addition, antibodies may only work with one technique; for example IgM antibodies may be suitable for immunofluorescence but not immunocytochemistry, as it is the case for FMC7 and anti-CD57. It should be appreciated, however, that different antibodies directed against a 'cluster of differentiation' may identify the same, or different epitopes, on a specific molecule. Various anti-CD34 antibodies, for example, react with different sites on CD34 and, as a result, have different binding

properties (Lansdorp, Dougherty & Humphries 1989). This may account for variation in results when the same sample has been analysed by different laboratories.

Antibody controls

Appropriate antibody controls must always be included in the investigative panel. It is not appropriate to use mouse immunoglobulin containing all the immunoglobulin classes as increased binding may be due to one subclass only. Therefore both antibody class (including subclass and isotype) and fluorochrome matched immunoglobulins should be used to determine the degree of non-specific binding. This is particularly important for setting positive/negative fluorescence thresholds on the flow cytometer or, if using ultraviolet microscopy or immunocytochemistry techniques, to provide a visual background staining level. For small volume samples it may be necessary to use a single control reagent (composed of a cocktail of relevant antibodies). Such reagents, however, do not allow the identification of the antibody in the event of non-specific binding. It is important to stress that the control antibody should be appropriately matched to the concentration and fluorochrome : protein ratio of the test antibody.

Antibody concentration

Antibodies from the same manufacturer can exhibit batch-to-batch variation. In addition, some control antibodies are not marketed at the same concentration as the test antibody. Ideally the F : P ratio (fluorochrome : protein ratio) should be 1 : 1. Many available reagents have high F : P ratios and are better avoided if alternatives exist (over-conjugation can produce highly charged acidic species which tend to stick non-specifically to cells, especially if they are fixed). It is important to establish the correct working dilutions for every new antibody, including those from different batches, for each detection system. Data from the UK NEQAS show that laboratories use a wide variety of antibody dilutions, even when antibodies are obtained from the same source. Ideally the 'titre value' should be determined for every antibody and this is of particular value in the investigation of suspected non-specific binding. Titre value is defined as the amount of antibody that is required to saturate the maximum number of antigen binding sites on a selected cell population. This is measured by the median positive fluorescence channel for the different antibody dilutions. The saturation, or titre value, is obtained by plotting the median peak channel fluorescence (MPCF) against antibody concentration, and is the point at which the MPCF plateaus (see below). The saturation value should be constant over at least two dilutions and curves which fail to plateau are usually the result of non-specific binding. It is important, when using indirect techniques, that each antibody is diluted to a pre-determined level so as to obtain maximum sensitivity and specificity. Under such circumstances a 'chequerboard' titration is the recommended procedure (see below). Clone and batch numbers of all reagents must be documented.

Storage

Storage conditions and expiry dates must be followed. Repeated freezing and thawing of purified or conjugated antibodies should be avoided, as this can lead to both antibody aggregation, resulting in non-specific binding, and to fluorochrome degradation and loss of antibody activity. In addition, repeated freeze/thawing results in loss of antibody and fluorochrome activity. A common error is the failure to mix reagents adequately following thawing, leading to increased concentration of antibody at the bottom of the tube or vial. Phycoerythrin-conjugated antibodies should not be frozen.

Fixatives

The UK NEQAS in Leucocyte Immunocytochemistry has highlighted the effect that different fixatives may have on antigen detection (Forrest & Barnett 1989). Recent trials have repeatedly demonstrated that CD3, CD5, CD13 and CD33 antigens are best preserved by using cold pure acetone fixation. Laboratories using formalin containing fixatives (especially greater than 0.5%) may miss these diagnostically important antigens. Cold pure acetone fixation, therefore, is recommended for all antigen detection.

Cell concentration

Cell separation techniques should involve standardization of final cell counts (10^6 cells/ml, in phosphate-buffered saline).

Fc blocking

Blocking of Fc receptors is important, particularly when analysing leukaemias with a significant monocytic component (Lawlor *et al.* 1986). Such samples require pre-incubation with either rabbit or human serum prior to analysis. In general, Fc receptor affinity varies according to antibody class, with IgM antibodies exhibiting the least binding (degree of Fc binding increases as follows: IgM

IgG_1 IgG_{2a} and IgG_{2b}). In practice the preferential use of monoclonal IgG_1 antibodies is to be recommended. Care is required during the washing stages in indirect methods, as traces of the first antibody in the supernatant can form complexes with the second antibody and bind to Fc receptors on irrelevant cells. An additional precaution that can be taken during indirect immunofluorescence analysis is the use of fluorescent $(Fab)^2$ fragments of xenogenic anti-mouse immunoglobulin (i.e. Fc cleaved).

Quality control

Both internal and external quality controls should be undertaken when performing leukaemia, or lymphocyte subset, analysis. Internal quality control procedures can include immunophenotyping fresh, or frozen, known positive and negative cells. In addition, control cells and calibration beads are now available for evaluation of monoclonal antibody by flow cytometry, although primarily for lymphocytic antigens (e.g. Absolute Control [Ortho Diagnostic], CD-Chex [Streck Laboratories], Cyto-Trol [Coulter] and Sphero Beads (Dako]). Flow cytometer users should also quality control linearity of fluorochrome amplification, as well as fluorescence intensity by means of commercial calibrants (e.g. Calbrite [Becton-Dickinson], Immuno-Brite [Coulter]). All calibration results should be documented to give an indication of machine performance and drift. A detailed discussion of analyser quality control is, however, outside the scope of this guideline and is adequately covered elsewhere (Lawry & Lowdell 1994). The above procedures do not replace the need for external quality assessment. This is essential, especially in laboratories in which techniques are run on an *ad hoc* or infrequent basis. This may involve participation in locally organized quality assurance schemes, in which small discussion groups and helpful advice can be generated, as well as participation in the UK NEQAS for Leucocyte Immunophenotyping.

Antibody selection

Despite the experience of a number of QC schemes worldwide, there is no consensus as to the best antibody within a CD for diagnostic use. Such evaluations would require multi-site studies, and the development of reference materials that express the antigens of clinical importance. Research in this area is currently under way, although the problem should not be underestimated.

Appendix. Protocol for antibody evaluation

Ideally every antibody should be titrated against each sample requiring analysis. This is impracticable, both in terms of time and resources. In practice the following procedures should be undertaken.

Flow cytometry

Direct technique Determine the optimum antibody dilution using a sample (normal or leukaemic) known to express the relevant antigen, ideally at the same density. In the case of the whole blood technique different volumes of

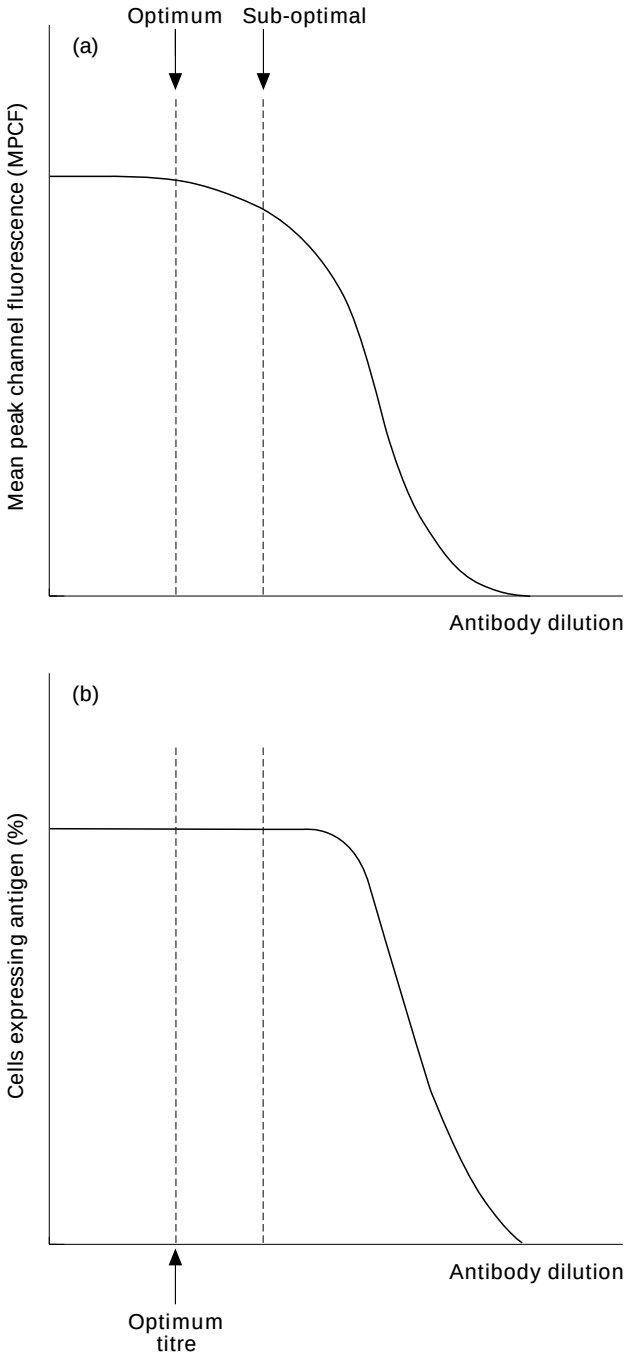

Figure 1. Determination of optimum antibody dilution.

directly conjugated antibody are added to a fixed volume of blood, to give rise to doubling dilutions of antibody. The sample is then analysed and two graphs plotted:

(i) Median peak channel fluorescence (MPCF) against antibody dilution (linear/log).
(ii) Percentage cell positivity against antibody dilution (linear/log)

The optimum antibody dilution is that which lies on the plateau area of both plots, i.e. a dilution that results in maximal fluorescence and cell positivity (see Figure 1). Failure to achieve a plateau effect in either plot suggests non-specific binding.

Indirect technique To determine the optimum dilution of both the primary and secondary antibodies use a chequerboard analysis. The final working concentrations are the conditions that give satisfactory maximum fluorescence and cell positivity counts when plotted as above.

Microscopy: immuno-enzymatic/fluorescence techniques

(a) Optimum antibody dilution is dependent on observer subjectivity, since it depends on the visual comparison of positivity against the background levels of negative controls. Ensure accurate pH of buffers, as the fluorescence signal can be quenched with a pH above and below 7.2. Controls for specificity of anti-TdT reagents should include cytospin preparations from known cases of acute lymphoblastic leukaemia (positive control) and acute myeloid leukaemia (negative control).

(b) Known positive and negative controls are required. The dilution of the first layer antibody should be determined by serial dilutions. The working dilutions of the second and third layer antibodies should be those recommended by the manufacturer.

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